

Anti-*Candida* Activity of Geraniol: Effect on Hydrolytic Enzyme Secretion and Biofilm Formation

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Existing antifungal drugs available today are low in efficacy, high in toxicity and cause multidrug resistance. Anti-*Candida* potential of geraniol, an acyclic monoterpene alcohol was evaluated and secretion of hydrolytic enzymes, adherence and biofilm formation were studied as virulence attributes. XTT reduction assay, inverted light microscopy and scanning electron microscopy (SEM) were used to study the effect of geraniol on biofilm formation. *In vitro* secretion of proteinases and phospholipases was observed on plates containing BSA and egg yolk, respectively. Geraniol showed minimum inhibitory concentration (MIC) of 130 µg/ml for *C. albicans* and *C. glabrata* and 80 µg/ml for *C. tropicalis*. Inhibition of biofilms was observed at sub-MIC values of geraniol. Proteinase secretion decreased in *C. albicans*, *C. tropicalis* and *C. glabrata* by 32.5-38.7%, 46.51%, 31.83%, respectively at MIC of geraniol. The clinical sensitive *C. albicans* D-27 and resistant S-1 showed 36.95% and 57.53% proteinase inhibition, respectively. Only minor inhibitory effects of geraniol were observed on phospholipase secretory activity. At MIC, the standard and resistant *C. albicans* strains showed 10-20% reduction in phospholipase inhibition, while the clinically sensitive strain showed an inhibition of 33%. Results show that geraniol interferes with the initial steps of fungal virulence and biofilm formation and can be used in the management and treatment of both topical and systemic *Candida* infections.

Keywords: *Candida*; geraniol; biofilms; proteinases; phospholipases.

Bloodstream *Candida* infections (Candidemia) become detrimental in immune-compromised patients and those in intensive care unit (ICU). The overall increase in incidence ranges from 75-400% depending on the size of the hospitals¹.² Having high mortality rates and treatment costs, early investigation and management is essential

to control these persistent and increasingly drug resistant fungal pathogens. *Candida albicans* is the most frequently isolated species but non-*albicans* species like *C. tropicalis* and *C. glabrata* are also to blame for nosocomial threats in mildly immunocompromised patients^{2, 3}. The virulence factors that contribute to *Candida* pathogenesis include biofilm formation, phenotypic switching, adhesion to host tissues, hyphal formation, and secretion of hydrolytic enzymes⁴. The yeast form is associated with dissemination in blood,

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whereas the hyphal form is responsible for host tissue infiltration during early stages of infection and hence crucial for pathogenesis. Virulence in *C. albicans* is related to extracellular secretion of hydrolytic enzymes, mainly phospholipases and proteinases. Phospholipases are responsible for various aggressive and defensive actions on the host leading to tissue invasion and cell membrane remodelling. Proteinases contribute to pathogenesis by degrading or modifying target epithelial and mucosal barrier proteins and ligands on the yeast surface, thus allowing proper adherence of the fungus to host tissues⁵.

Candida cells adhere, colonize and form biofilms on medical implants such as blood and urinary catheters, dentures, artificial joints and voice prosthetics, causing high risk of infection in patients. Biofilm associated cells are much more resistant to existing antifungal drugs than planktonic cells due to complex biofilm structures, extracellular matrix and up-regulation of efflux pumps^{6, 7}. Drug resistance associated with the biofilm mode of growth is multifactorial and involves production of persister cells, quorum sensing, production of extracellular matrix and drug binding and stress responses^{8, 9}.

Plants and plant products have exhibited immense potential as antimicrobials^{10, 11}. Terpenoids are a major class of natural compounds that have shown antifungal properties¹¹⁻¹⁵. Geraniol, an acyclic monoterpene alcohol (Fig 1) is the major component of numerous essential oils like geranium oil, palmrosa oil, ninde oil, rose oil and citronella oil¹⁶. It possesses insecticidal, antihelminthic, antibacterial, antioxidant, anticancer and anti-inflammatory activities¹⁶⁻¹⁸ and is a good repellent for malarial, filarial and yellow fever vectors¹⁹. Recently, we showed that geraniol alters fungal cell morphology, significantly reduces ergosterol levels and inhibits glucose induced H⁺ efflux suggesting that it disrupts cell membrane integrity by interfering with ergosterol biosynthesis and H⁺ ATPase activity¹⁸. It has also been shown that geraniol causes mitochondrial dysfunction, impairs iron homeostasis and genotoxicity^{18, 20}. This natural compound demonstrated negligible toxicity to human erythrocytes and hence is a good candidate to be studied as a potential antifungal drug. In the present study we have studied the effect of geraniol on hydrolytic enzymes secretion and biofilm

formation as virulent factors on both sensitive and fluconazole (FLC)-resistant *C. albicans* strains along with standard strains of *C. albicans*, *C. glabrata* and *C. tropicalis*. To our knowledge the present study is the first one to show the effect of geraniol on the secretion of proteinases and phospholipases. These hydrolytic enzymes play a crucial role in facilitating adhesion, tissue damage and evasion of host immune responses²¹.

MATERIALS AND METHODS

Strains and media

C. albicans (ATCC 10261 and ATCC 90028), *C. glabrata* ATCC 90030 and *C. tropicalis* ATCC 750 were the standard strains used in the present study. In addition, *C. albicans* D-27 (FLC sensitive) and *C. albicans* S-1 (FLC resistant) were isolated from vaginal swabs of diabetic patients obtained from Safdarjung Hospital, New Delhi, India. Strains showing MIC \geq 64 μ g/ml for FLC were considered resistant. All the strains were maintained on YEPD (yeast extract 1%: peptone 2%: dextrose 2%: agar 2.5%) plates at 4°C. All the chemicals were of analytical grade and were procured from E. Merck (India). The media components were procured from Himedia (India) while geraniol was purchased from Aldrich (Germany).

Broth dilution method

Minimum inhibitory concentration (MIC) values of geraniol for the *Candida* strains were determined by the broth dilution method²². MIC was defined as the lowest concentration that causes 90% decrease in absorbance in comparison to that of the control (without test compound). After recording MIC values, 20 μ l samples from all optically clear tubes (no growth) along with the last tube showing growth were sub-cultured on YEPD agar plates. The plates were incubated at 35°C for a minimum of 3 days, until growth was clearly visible in the control samples, and MFC values were calculated as the lowest concentration of the test compound for which there was no visible growth²³. Results were calculated as a mean of the two separate experiments done in triplicate.

Spot Assay

For studying susceptibility of geraniol to *Candida* cells²⁴, cells were grown overnight in YEPD media at 37°C. Cell culture was suspended

into normal saline (optical density of 0.1 at 600 nm). For the assay, 5 µl of fivefold serial dilution of each *Candida* strain was spotted on YEPD agar plates in the absence (control) and presence of ¼ MIC, ½ MIC, MIC, 2 MIC of geraniol. Difference in growth was observed after 48 hours of incubation at 30°C. The solvent (1% DMSO) showed no effect on cell growth (data not shown).

In vitro Candida sensitivity to geraniol

Candida cells (10⁵ cells/mL) were inoculated into YEPD agar at 40°C and poured into 90-mm petri plates¹⁶. Sterile filter discs (4 mm) laden with the test compounds at different concentrations (3 MIC, 4 MIC and 5 MIC) were placed on agar plates. The diameter of inhibition zones (ZOI) was measured after 2 days. FLC (10 µg/mL) was also included as a positive control. The sensitivity index (SI) was calculated for all the three *Candida* species as ZOI (mm)/concentration of the antifungal compound (mg/mL). The SI values are shown as mean ± SD²⁵.

WST1 Cytotoxicity assay

Cell suspensions (1 × 10⁵ cells/ml) were cultured in 96-well plate with a final volume of 100 µl/well along with appropriate concentrations of geraniol (≤ MIC) for 24 h followed by 2 h incubation at 37°C with 10 µl of WST-1/CEC dye. Plates were gently shaken to mix the well constituents. The absorbance was recorded at 450 nm using a microplate Reader (BIORAD iMark, US) and reference was set at 655 nm. The absorbance values for culture medium were subtracted from the assay results. Each reading was taken in triplicate. Percentage cytotoxicity was calculated using the following equation:

$$\% \text{ Cytotoxicity} = [100 \times (\text{cell control} - \text{test value}) \div (\text{cell control})]$$

Proteinase assay

Candida cells were inoculated and incubated for 18 hrs at 37°C in 5 ml YEPD media. Cells were centrifuged for 5 min at 3000 rpm and the pellets were re-suspended in saline, after washing twice for removing the residual culture medium. The cell suspensions, after standardization to a MacFarland 0.5 index, were exposed to desired concentrations of geraniol. After overlaying 2 µl on agar surface (agar 2%; BSA fraction V 0.2 g; yeast nitrogen base w/o amino acids; ammonium sulphate 1.45 g; glucose 20 g; sterile water to 1

L) at equidistant points, the plates were incubated for 2-3 days at 37°C. Proteinase secretion was estimated as Pz values, calculated by measuring the diameters of degradation zones and then by dividing the diameter of the colony by the diameter of the colony together with zone of clearance^{23, 26}.

Phospholipase assay

Cell suspensions (MacFarland 0.5 index) were exposed to geraniol at MIC and sub-MIC and 2 µl pipette on agar peptone media (agar 2%, peptone 10g, glucose 30g, NaCl 57.3g, CaCl₂ 0.55g, distilled water added to 0.9 L) enriched with 10% (v/v) egg yolk emulsion (HiMedia). The plates were incubated for 2-4 days at 37°C and phospholipase secretion was estimated by measuring precipitation zones. Pz values were calculated as mentioned above^{26, 27}.

Adhesion assay and Biofilm formation

Cell suspension (100 µl of 10⁷ cells/ml) in RPMI 1640 media was inoculated into each well of a sterile, 96-well microtiter plate²⁸. Geraniol was added to each well at desired concentrations except in control. The plates were incubated at 37°C for 90 min in a shaker at 75 rpm to allow adherence of cells. Each well was gently washed twice with PBS (150 µl) to remove non-adherent cells. The adherent cells were observed using Motic AE31 (Germany) Inverted microscope²⁹. For biofilm formation, cell suspensions (1 × 10⁷ cells/ml in RPMI 1640) were inoculated into wells and incubated at 37°C for 1h. After the initial 1h adhesion period, fresh RPMI 1640 media, containing desired concentrations of geraniol were added to the plate wells containing adherent cells and incubated for 24 h at 37 °C. The adherent cells and biofilms were estimated using a semi-quantitative XTT reduction assay.

XTT- reduction assay

2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay is based on the reduction of XTT tetrazolium formazan by active mitochondria in *Candida* cells²⁸. Briefly, pre-formed *Candida* biofilms were washed with PBS. XTT (300 mg/L) was added to 200 µl PBS. An aliquot of XTT (100 µl) was then added to each pre-washed biofilms and control wells (to measure background XTT levels). The plates were incubated in dark for 5 h at 37°C without shaking. After 2min agitation the colorimetric change was observed at 450 nm (a direct correlation to metabolic activity of biofilms)

using a microtiter plate reader (BIO-RAD, iMark, US). The wells containing PBS only or PBS plus XTT were used as blank. Biofilms were simultaneously examined using scanning electron microscopy (SEM).

Scanning Electron Microscopy

Post geraniol treatment, the biofilms formed as described above, were washed with PBS and fixed overnight in 2% glutaraldehyde (v/v) in PBS. The samples were washed twice in PBS and then placed in 1% osmium tetroxide for 30 min. Subsequently, the samples were dehydrated in a series of ethanol washes (70% for 10 min, 95%

for 10 min, 100% for 20 min), and finally dried in a critical point dryer. The samples were then coated with gold/palladium (40%/60%). Biofilms were observed under Scanning electron microscope (ZEISS), following the given analytical conditions EHT = 3.00 kv, WD = 9.0mm, signal A = SE1, Mag = 3.07 K X.

Statistical analysis

All experiments in the present study were performed in triplicate and results were expressed as mean \pm standard deviation (SD). Results were analysed employing Students t-test and $P < 0.05$ was considered statistically significant.

Table 1. Minimum inhibitory and fungicidal concentrations for geraniol against *Candida* strains

| <i>Candida</i> strains | Type | MIC ($\mu\text{g/ml}$) | MFC ($\mu\text{g/ml}$) |
|-------------------------------|---------------------------------------|-----------------------------|-----------------------------|
| <i>C. albicans</i> ATCC 10261 | Standard (American Type Cell Culture) | 130 | 160 |
| <i>C. albicans</i> ATCC 90028 | | 130 | 160 |
| <i>C. glabrata</i> ATCC 90030 | | 130 | 160 |
| <i>C. tropicalis</i> ATCC 750 | | 80 | 140 |
| <i>C. albicans</i> D-27 | Clinical FLC-sensitive | 80 | 140 |
| <i>C. albicans</i> S-1 | | 175 | 250 |

Fluconazole (FLC) showed an MIC of 10 $\mu\text{g/ml}$ for all standard strains, 6 $\mu\text{g/ml}$ for FLC-sensitive and 125 $\mu\text{g/ml}$ for FLC-resistant *Candida* strains

Table 2. *In vitro* *Candida* sensitivity to geraniol as determined by disc diffusion assay. Each strain was tested in triplicate. Sensitivity Index (SI) is expressed as mean \pm SD and was calculated as the ratio of diameter of ZOI (mm) to concentration of test compound (mg/ml)

| <i>Candida</i> species | Test compound | Sensitivity Index (SI) |
|-------------------------------|----------------------------|------------------------|
| <i>C. albicans</i> ATCC 10261 | 3 MIC | 2.2703 \pm 0.017 |
| | 4 MIC | 2.37 \pm 0.06 |
| | 5 MIC | 2.52 \pm 0.052 |
| | FLC (10 $\mu\text{g/ml}$) | 5.68 \pm 0.06 |
| <i>C. glabrata</i> ATCC 90030 | 3 MIC | 1.935 \pm 0.091 |
| | 4 MIC | 2.12 \pm 0.061 |
| | 5 MIC | 2.48 \pm 0.080 |
| | FLC (10 $\mu\text{g/ml}$) | 4.866 \pm 0.55 |
| <i>C. tropicalis</i> ATCC 750 | 3 MIC | 2.311 \pm 0.062 |
| | 4 MIC | 2.466 \pm 0.0353 |
| | 5 MIC | 2.65 \pm 0.027 |
| | FLC (10 $\mu\text{g/ml}$) | 5.435 \pm 0.065 |

RESULTS AND DISCUSSION

C. albicans and *C. glabrata* comprise nearly 70-80% of *Candida* species isolated from patients suffering from candidemia or invasive candidiasis. Among the non-*albicans* species, the highest mortality is linked with *C. tropicalis* and *C. glabrata* [30]. *C. tropicalis*, which comprises 6-12% of total *Candida* species, is apparently the most prevalent in India³⁰ and is considered the causative agent of candidemia in cancer patients³¹. Hence these three *Candida* species were used to study the fungal virulence factors in the presence of geraniol (Fig. 1), a monoterpenoid alcohol, and one of the main components of several essential oils. In a previous study, we evaluated its anti-*Candida* potential and showed that it disrupts cell membrane integrity by interfering with ergosterol

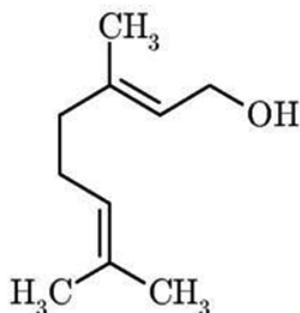


Fig. 1. Structure of geraniol, a monoterpenoid alcohol

biosynthesis and H⁺-ATPase activity¹⁶. Here we further study the effect of this natural compound on secretion of hydrolytic enzymes (proteinases and phospholipases), adhesion and biofilm formation in both sensitive and resistant clinical strains of *C. albicans* and two other non-*albicans Candida* species.

Table 1 shows the MIC and MFC values of geraniol for the *Candida* strains studied here. The MIC of geraniol for *C. albicans* (ATCC 10261 and ATCC 90028) and *C. glabrata* (ATCC 90030) was 130 µg/ml while for *C. tropicalis* (ATCC 750) it was only 80 µg/ml. The corresponding MFC values for these standard strains were slightly higher - 160 µg/ml for *C. albicans* and *C. glabrata*, and 140 µg/ml for *C. tropicalis*. Interestingly, geraniol showed more sensitivity towards the clinical sensitive strains than the standard strains. FLC-sensitive *C. albicans* D-27 showed an MIC of 80 µg/ml while FLC-resistant *C. albicans* S-1 gave a higher value of 175 µg/ml. Although resistant *Candida* strains showed less sensitivity in comparison to standard and sensitive strains, still the antifungal potential was high enough to perform further studies. The MFC values were again only slightly higher with 140 µg/ml in FLC-sensitive and 250 µg/ml for FLC-resistant clinical strains of *C. albicans*. FLC, the conventional antifungal drug showed an MIC of 10 µg/ml for standard *Candida* strains. The value was 6 µg/ml for FLC-sensitive and 125 µg/ml for FLC-resistant *C. albicans* as shown in table 1.

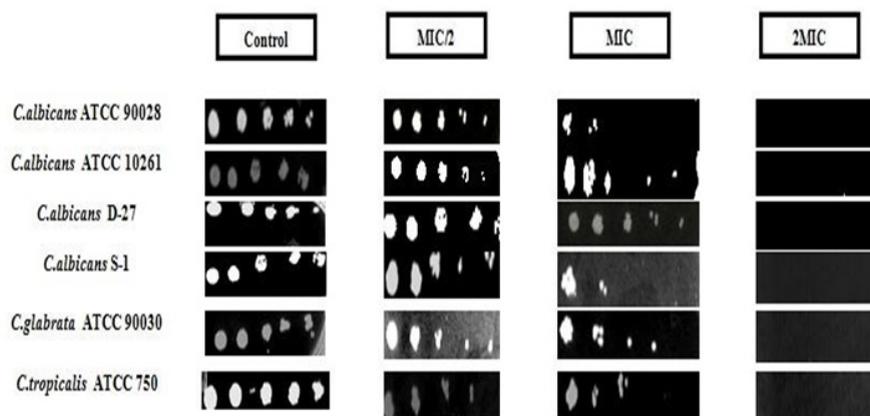


Fig. 2. Spot assay showing susceptibility of *C. albicans* (ATCC 90028, ATCC 10261, sensitive strain (D-27), resistant strain (S-1), *C. glabrata* and *C. tropicalis* to varying concentrations of geraniol. Control has no test compound

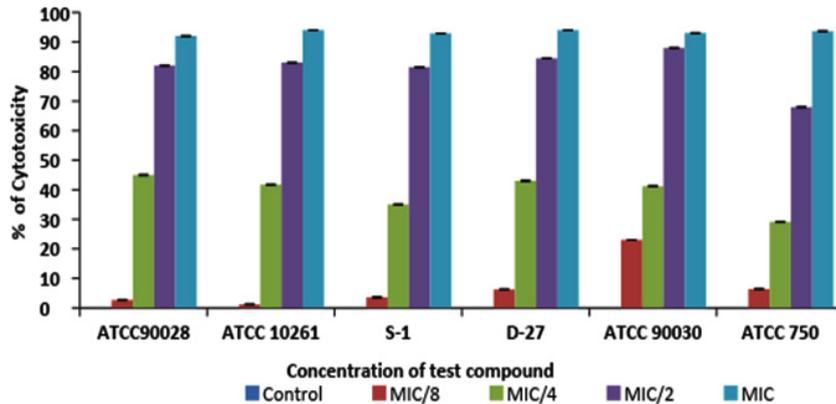


Fig. 3. The cytotoxic activity of geraniol against *C. albicans* (ATCC 90028 & 10261), *C. albicans* D-27, *C. albicans* S-1, *C. glabrata* (ATCC 90030), *C. tropicalis* (ATCC 750). Error bar lines represents \pm SD to the mean value from 3 different recordings

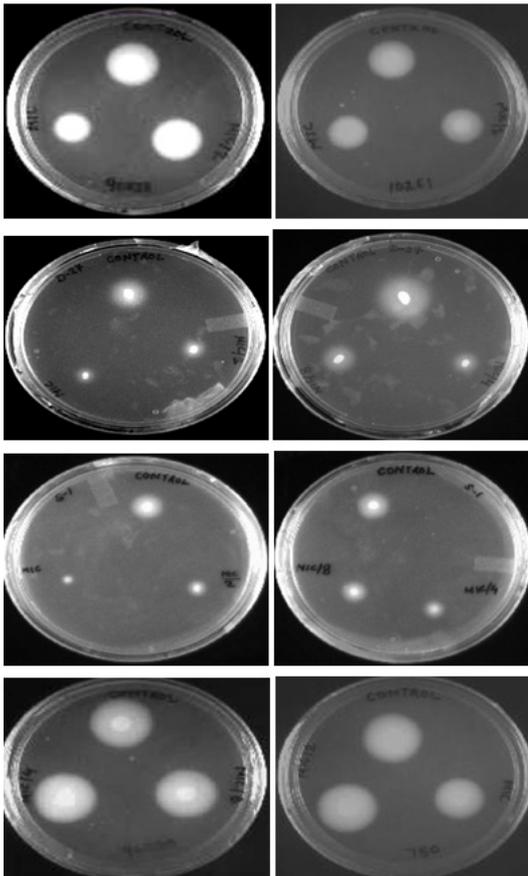


Fig. 4. (a) BSA agar plates showing the proteinase secretion in *C. albicans* ATCC 90028 and 10261 (A, B), *C. albicans* D-27 (C, D), *C. albicans* S-1 (E, F), *C. glabrata* ATCC 90030 (G), *C. tropicalis* ATCC 750 (H), in the presence of geraniol at 1/8 MIC, 1/4MIC, 1/2 MIC and MIC

Antifungal susceptibility was also investigated on solid media by spot assay (Fig. 2) as one of the confirmatory methods. *Candida* cells were grown on YEPD agar for 48 h at 30°C. Five spots in horizontal direction represent the serial dilution of cell culture. There is a significant reduction in the growth of *Candida* cells, which is concentration dependent. At MIC, the strains showed little or no proliferation at fourth dilution. *C. albicans* did not show any proliferation even at third dilution. No growth was observed in all the tested strains at 2 MIC, strongly recommending geraniol an antifungal agent. The results summarized in table 2 gives an estimate of the sensitivity of the strains for the antifungal compound. All three types of standard *Candida* strains showed sensitivity evident from zone of clearance (results not shown here). Sensitivity index (SI) was calculated as the ratio of the diameter of ZOI (mm) to the concentration of test compound (mg/ml). Sensitivity of geraniol increased with increasing concentration and *C. tropicalis* was more sensitive than *C. albicans* followed by *C. glabrata*. In all three cases sensitivity was less than that of FLC but the results are still encouraging as the test compound has low toxicity and is fungicidal in nature while FLC is fungistatic and leads to resistance.

WST1 cytotoxic assay was performed to check yeast cell viability in the presence of geraniol, by a colorimetric method based on the principle of the metabolizing activity of mitochondrial succinic oxidase, cytochrome P-450 systems and

flavoprotein oxidase of living fungal cells^{32, 33}. This leads to the conversion of XTT to formazan, which is a water soluble product and hence can be easily measured in supernatants. The generation of the dark yellow coloured formazan is measured at 420-480 (optimal at 440 nm) and is directly correlated to cell number. This way biofilms can be studied as intact structures, and can be observed for drug susceptibility without disruption of their structures. Cells were cultured in a microplate and then incubated with the reagent WST-1 and the assay was monitored spectrophotometrically. It showed $\geq 90\%$ cytotoxicity at MIC values for all tested strains (Fig. 3). This correlates well with the growth data presented in Fig 2. At concentrations of $\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC, average range of cytotoxicity was 68-88% and 30-45%, respectively in all three species. The above mentioned study revealed that

geraniol shows high cytotoxic activity and affects the metabolic activities of yeast cells leading to decreased growth and viability, which can be explored with further studies.

Extracellular proteinases and phospholipases are important for the invasion of host tissues by *Candida* cells. Strains were tested for proteinase activity in the presence and absence of geraniol at MIC and sub-MIC concentrations (1/2 MIC, 1/4 MIC and 1/8MIC). Fig. 4 (a) shows degradation zones produced on solid media as a result of proteinase secretion by *Candida* species. At MIC, geraniol showed 32.5-38.7%, 46.51%, 31.83% proteinase inhibition in *C. albicans* (ATCC 10261 and ATCC 90028), *C. tropicalis* ATCC 750 and *C. glabrata* ATCC 90030 respectively. The clinical strains, *C. albicans*D-27 and S-1 showed 36.95% and 57.53% proteinase inhibition,

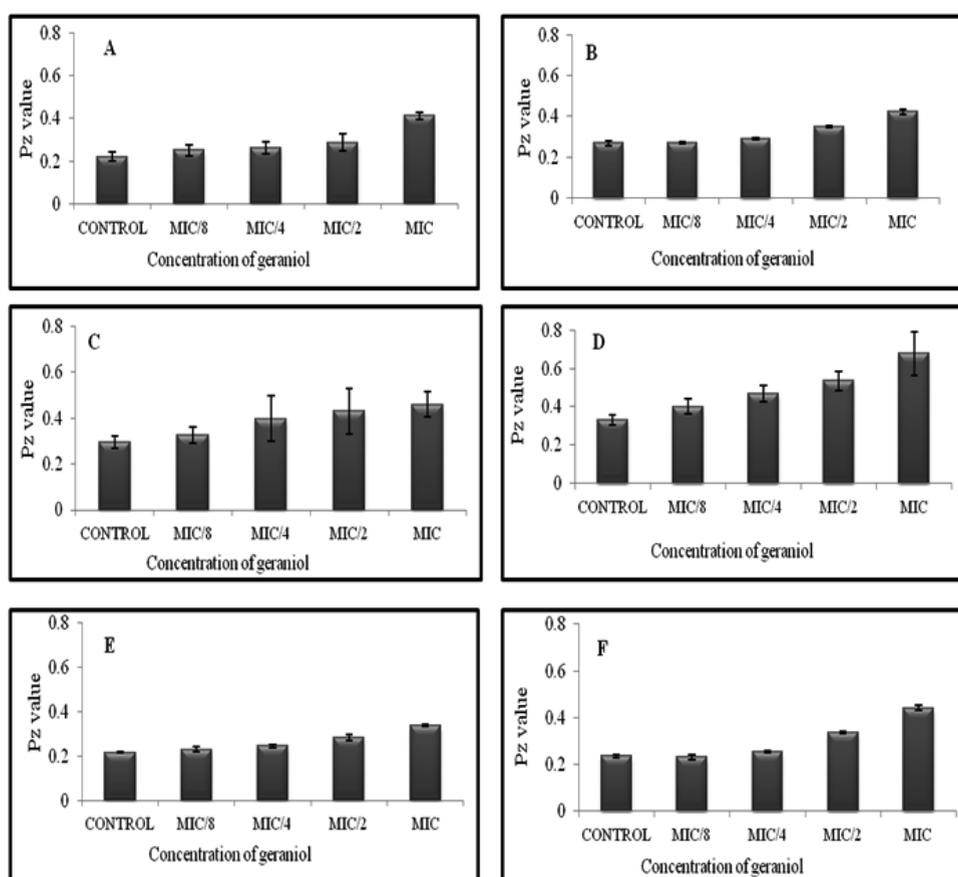


Fig. 4. (b). Effect of geraniol on proteinase secretion by *C. albicans* ATCC 90028 and 10261 (A and B), *C. albicans* D-27 (C), *C. albicans* S-1 (D), *C. glabrata* ATCC 90030 (E), *C. tropicalis* ATCC 750 (F). Error bars represent \pm SD to the mean value from 3 different readings

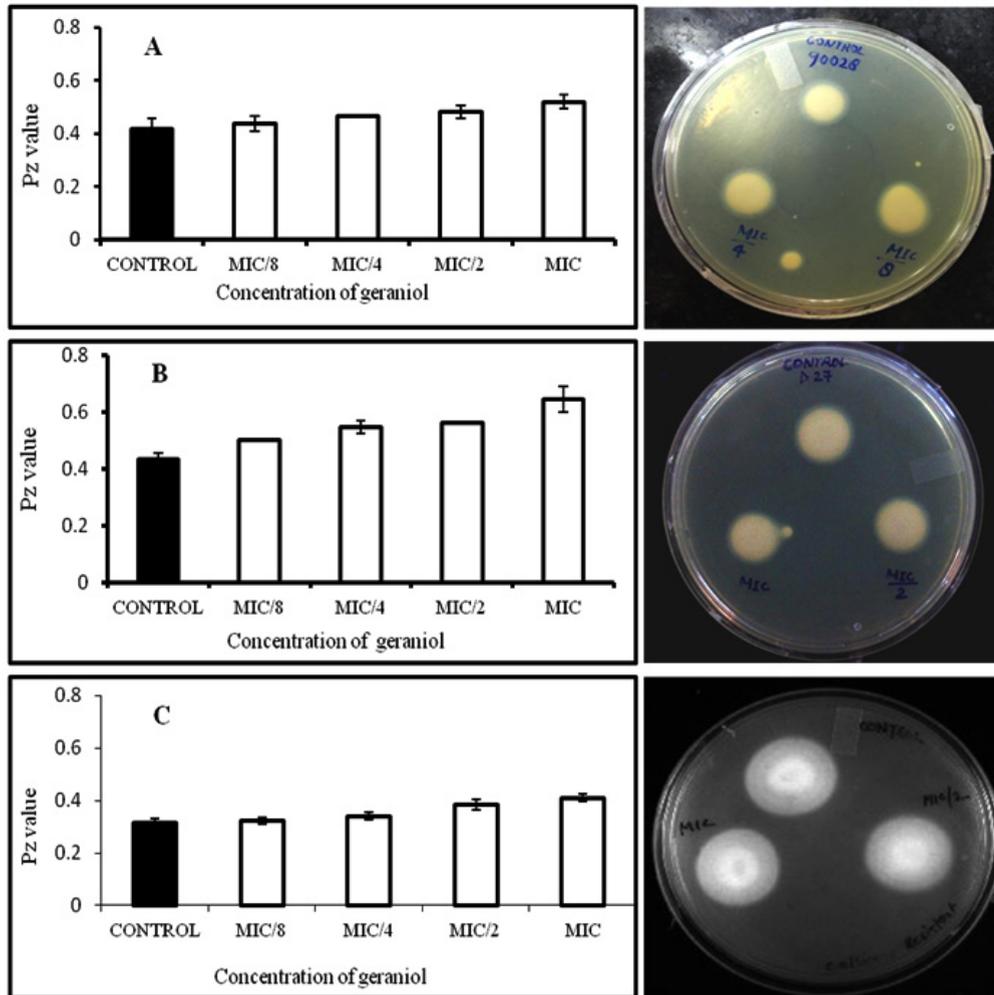


Fig. 5. Effect of geraniol on phospholipase secretion in *C. albicans* ATCC 90028 (A), *C. albicans* D-27 (B), *C. albicans* S-1 (C) in the presence of different concentrations of geraniol (1/8 MIC, 1/4 MIC, 1/2 MIC and MIC)

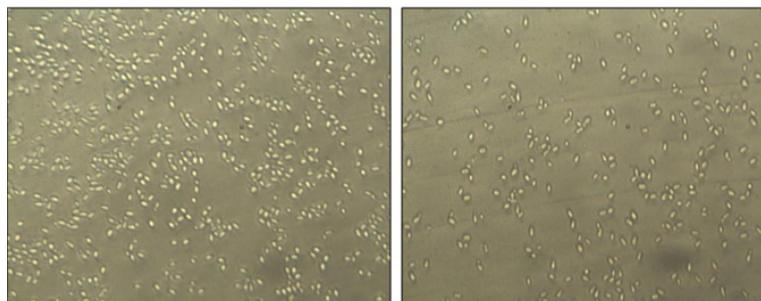


Fig. 6. Inverted microscopic pictures of adhesion, showing the decrease in number of adherent *C. albicans* (ATCC 10261) cells treated with MIC of geraniol (B), while cells in control (A) show a large number of adherent cells in the absence of geraniol

respectively. At 1/2 MIC, this compound showed enzyme inhibition in the range of 30-40% for all tested strains (Fig 4b). Results showed that at 1/4 MIC and 1/8 MIC, geraniol does not have any significant effect on secretion of proteinases. A decrease in secretion of proteinases under the effect of geraniol points towards the less virulent nature of *C. albicans* and non-albicans species in the presence of this natural compound. FLC-resistant isolates showed even lower secretory activity of proteinases in comparison to standard and sensitive strains, indicating high antifungal potential of geraniol in the resistant strain.

The secretory activity of phospholipases was not displayed by all the strains. Secretion was observed only in *C. albicans* ATCC 10261 and *C. tropicalis* ATCC 750 while *C. glabrata* ATCC 90030 failed to secrete this hydrolytic enzyme. Our results correlate with previously reported data, where screening of 41 *Candida* isolates showed phospholipase secretion in only 79% *C. albicans*, while none of the strains of *C. glabrata*, *C. tropicalis*, *C. parapsilosis* secreted the enzyme³⁴. Hence, only secretory activity of *C. albicans* ATCC 90028, D-27 and S-1 have been shown here. Our data shows that the inhibitory effect

of geraniol on phospholipase secretory activity was not as significant as on proteinase secretion. At MIC, geraniol showed 10%, 20% and 33% reduction in phospholipase secretion in case of *C. albicans* ATCC 90028, *C. albicans* S-1 and *C. albicans* D-27 respectively (Fig 5). The percentage inhibition for *C. albicans* ATCC 10261 was 15-21% (Figure not shown). Secretion of phospholipases is not significantly inhibited in comparison to proteinases, which may be due to the reason that geraniol has inhibitory effect on SAP genes rather than PLB genes¹.

Fungal cell adherence to host tissues is the initial step crucial to the process of biofilm formation. The amount and the characteristics of biofilms were microscopically studied with the semi-quantitative colorimetric technique based on XTT-reduction assay. Microscopic analysis and colorimetric readings revealed that the formation of hyphae and biofilms was inhibited by geraniol in a concentration dependant manner. Fig 6 shows microscopic pictures of cell adherence on polystyrene surface. The control (6A) shows a large number of adherent cells in comparison to the treated case (6B). More than 60% microbial infections in humans are biofilm related,

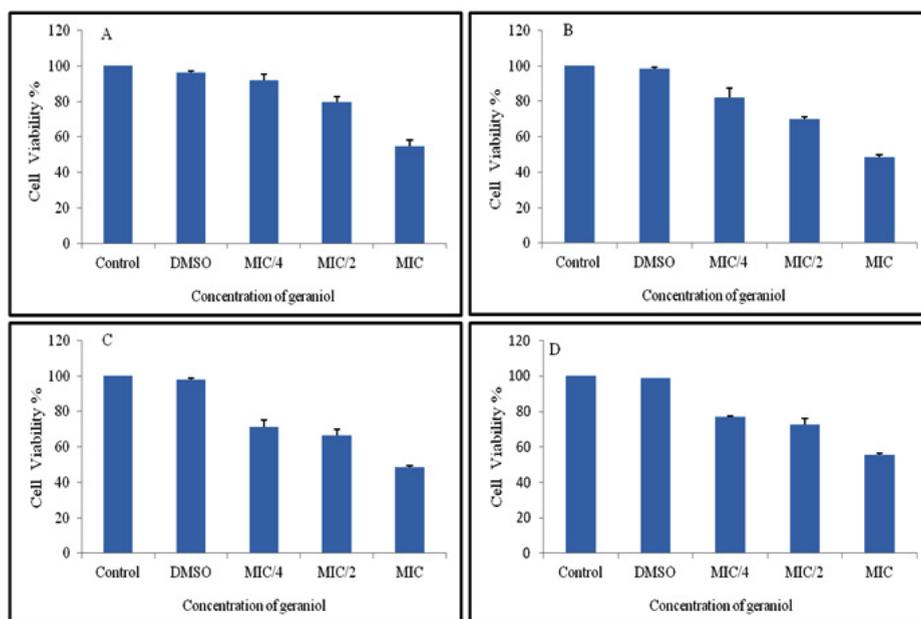


Fig. 7. Effect of varying concentrations of geraniol (MIC, 1/2 MIC, 1/4 MIC) on adhesion in *C. albicans* ATCC 90028 (A), *C. albicans* ATCC 10261 (B), *C. albicans* FLC sensitive D-27 (C) and FLC resistant *C. albicans* S-1(D). Cell viability was estimated using the XTT reduction assay after 1 h incubation. 1% DMSO was also used as a control

where *C. albicans* biofilm is a major virulence factor responsible for high risk of mortality in some patients. Chronic biofilms facilitate carcinogenesis by causing inflammation³⁵ and chronic mucocutaneous candidiasis is a risk for oral

cancer³⁶. The present study shows that adherence of *C. albicans* cells to polystyrene surface was significantly inhibited by geraniol. Adherence was inhibited by 45%, 38%, 52% and 44.6% in *C. albicans* ATCC 90028, *C. albicans* D-27 and *C.*

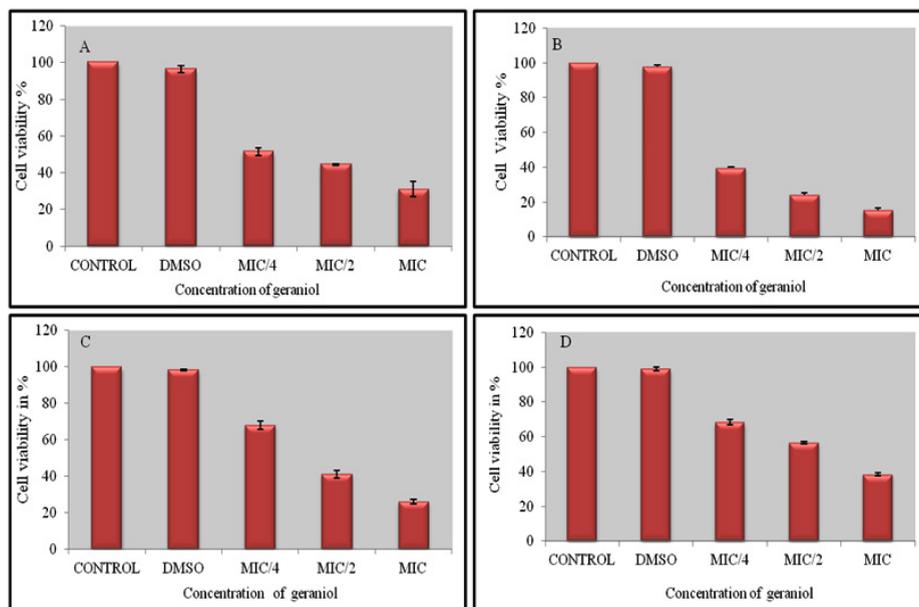


Fig. 8. The effect of varying concentrations of geraniol (MIC, $\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC) on biofilm formation in *C. albicans* ATCC 90028 (A), *C. albicans* ATCC 10261 (B), *C. albicans* FLC sensitive D-27 (C) and FLC resistant *C. albicans* S-1(D). Cell viability was estimated using the XTT reduction assay after 24 h incubation. 1% DMSO (control) had no effect on biofilms. Error bars represent \pm SD to the mean value from 3 different values

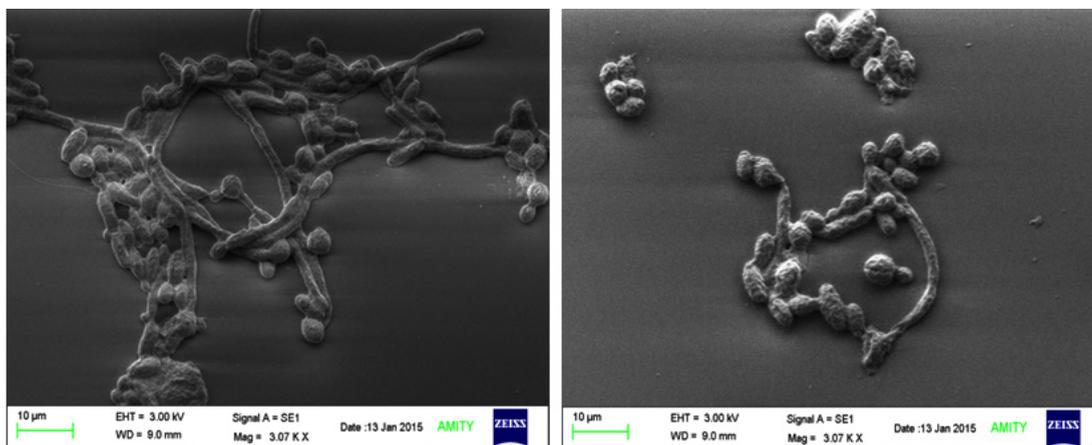


Fig. 9. Scanning electron micrographs of *C. albicans* ATCC 10261 biofilms grown for 24 h on silicon discs in the absence (A) and presence (B) of geraniol at MIC. In the absence of geraniol a dense population of yeast cells and hyphae could be seen while in the presence, a loss in the biofilm structural elements could be observed

albicans S-1 at their respective MIC values. At 1/2 MIC, inhibition was 20.54%, 33.72 and 27.4 % in the same order. In case of *C. albicans* ATCC 10261, cell adherence was inhibited by 50%, 30 % and 33 % at MIC, 1/2 MIC and 1/4 MIC, respectively (Fig. 7).

Biofilms formed by *C. albicans* are highly structured and composed of round, budding yeast cells along with pseudohyphae and hyphae encased within an extracellular matrix. Their formation comprises of four different stages that include adherence to host surfaces. This is the initial stage followed by proliferation and growth of pseudohyphae and hyphae. Finally the yeast cells disperse to seed biofilms to new sites in the host¹⁷. *C. albicans* associated biofilms are difficult to treat due to acquired resistance for existing antibiotics. Hence, a search for novel compounds against fungal biofilms is a big necessity now and natural compounds are good candidates as they are non-toxic, easily available and do not lead to multidrug resistance. Majority of biofilms are commonly formed by *C. albicans* rather than non-*albicans* *Candida* species³⁷. XTT reduction by the metabolic activity of cells is used to study the inhibitory activity of geraniol on biofilms. Exposure to geraniol at MIC, caused inhibition in biofilm formation of 71.8%, 75-79% and 63% respectively in *C. albicans* ATCC 90028, *C. albicans* D-27 and *C. albicans* S-1. Biofilm inhibition in the same strains at lower concentration of 1/2 MIC was ≤55%, ≤58% and 43%.

C. albicans ATCC 10261 showed 85% inhibition at MIC of geraniol followed by 1/2 MIC (79) and 1/4 MIC (60%) (Fig.8). *C. albicans* D-27 (FLC-sensitive) and *C. albicans* ATCC 10261 showed higher sensitivity towards adherence as well as biofilm formation when treated with varying concentration of geraniol. Inhibitory effect of geraniol on cell adherence and biofilm formation was dose dependent. Significant reduction in biofilm formation may be due to the inhibition of yeast to hyphal transition. The morphological transition provides dense structural integrity to biofilm complexes. Geraniol may be responsible for reducing cell adherence which is crucial for biofilm formation. Previous studies have also linked inhibition of yeast to hyphal transition to anti-biofilm activity in *C. albicans*¹⁸. The contribution of yeast to hyphal transition is

significant in the formation of biofilms and hence is responsible for the virulence and pathogenicity of this opportunistic fungus.

Figure 9 shows SEM visualization of biofilm formation by *C. albicans* on plastic cover slips. Cells treated with geraniol at MIC produced less dense biofilms, which were composed mainly of yeast cells and pseudohyphae. Poor biofilm architecture was observed in treated cells due to absence of true hyphae (8B). The control biofilms (8A) formed in the absence of geraniol were denser and constituted mainly true hyphae and pseudohyphae forming a fairly good architecture. The data was consistent with biofilm results estimated by XTT reduction assays.

In conclusion, our work demonstrates that geraniol exhibits potent antifungal activity against *Candida* species, inhibits biofilm formation, reduces cell adherence and decreases the secretion of hydrolytic enzymes especially proteinases. Results clearly suggest that geraniol is clinically significant and may be used in treating biofilm-associated *Candida* infections. Further research is required both *in vivo* and *in vitro* to validate the diverse mode of antifungal action of geraniol as a promising antifungal agent.

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